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Evaluation of a Sandwich Gene Probe Assay for Newcastle Disease Virus

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February1996

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NO. 1474*

EVALUATION OF A SANDWICH GENE PROBE ASSAY FOR NEWCASTLE DISEASE VIRUS

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Douglas E. Bader and Darrin Gray*

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February 1996

ABSTRACT

A sandwich gene probe assay was evaluated in comparison to a direct gene probe assay. The target sequence used in each of the assays was a 673 bp DNA fragment of the major nucleocapsid protein gene of NDV. In the direct probe assay, the 673 bp DNA fragment was labelled with digoxigenin and hybridized to unlabelled 673 bp target DNA. In the sandwich assay, the target DNA was detected using two probes. The primary probe was unlabelled, recombinant M13mp18 viral DNA containing the 673 bp gene fragment which hybridized to the 673 bp target DNA. The secondary probe was digoxigenin-labelled M13mp18 DNA which hybridized to the M13 sequences within the primary probe.

The sandwich assay resulted in detection limits similar to those demonstrated for the direct assay (10^5 molecules of purified target DNA) when molar probe concentrations for the two assays were around 20 pM. When molar probe concentrations in the sandwich assay were increased beyond this, sensitivity decreased and background problems due to non-specific binding became evident. Based on these results, the direct assay is the method of choice since the sandwich assay was no more sensitive than the direct assay, required more probe material and required additional time-consuming probe preparation steps.

EXECUTIVE SUMMARY

Title   Bader, D.E. and Gray, D. "Evaluation of a Sandwich Gene Probe Assay for Newcastle Disease Virus". *DRES Suffield Memorandum No.* 1474, 1996.

Introduction   Gene probe assays are an increasingly important technology in detection and identification of biological agents, as well as medical diagnostics. Gene probe assays use discrete sequences of nucleic acid (DNA or RNA), that are complementary to distinct regions of the genetic material being analyzed. Since nucleic acid is a component of all living material, probes can be devised and used against nucleic acid from essentially all sources including viruses, bacteria, plants or animals. In addition, gene probes can be used to identify genetic sequences that have been transferred naturally or by human design into foreign vehicles, for example, toxin genes cloned into innocuous organisms or infectious nucleic acid contained within man-made microcapsules.

Gene probe assays are conventionally performed as mixed-phase assays whereby the target DNA is bound to the solid phase and the probe hybridizes to the target in solution phase. We have previously developed a mixed-phase gene probe assay for Newcastle disease virus using nylon membranes as the solid phase. The gene probe used in each of these assays was a 673 bp DNA fragment of the major nucleocapsid protein gene of Newcastle disease virus (NDV). The 673 bp sequence was labelled with digoxigenin, a non-radioactive label, and used as a probe against unlabelled 673 bp DNA. This assay format is termed a direct assay since the bound target DNA is hybridized directly with a gene probe.

An alternative assay format, called a sandwich assay, has been described in the literature which uses two probe sequences. One probe sequence recognizes and binds to the target analyte and the other probe sequence recognizes and binds to the first probe sequence. The implication of the sandwich assay format is that it may allow for enhanced assay sensitivity

through the formation of networks (multi-labelled complexes) brought about through the overlapping of complementary regions of the two gene probes. We were interested in determining whether this sandwich assay format would result in greater sensitivities than the direct gene probe assay. This report describes such a study.

Results   The sandwich assay resulted in detection limits similar to those demonstrated for the direct assay (10^5 molecules of purified target DNA) when molar probe concentrations between the two assays were around 20 pM. When molar probe concentrations in the sandwich assay were increased beyond this, sensitivity decreased and background problems due to non-specific binding became evident.

Significance of Results   The sandwich assay did not offer any advantage over the direct assay with respect to sensitivity or ease of use. These results validate the use of the direct assay format as the method of choice for probe assays being developed at DRES.

Future Goals   The goal of this study and recent gene probe studies at DRES has been to develop a working knowledge of the utility of gene probes as tools for the identification of biological agents, with emphasis on assay design, detection limits and ease of use using the viral model Newcastle disease virus. We have gained experience in gene probe design using NDV as a model organism and will use this knowledge to design and procure gene probe sequences for biological agents of concern to the CF. We have and will continue to evaluate gene probe related technologies that improve upon our current capabilities with regards to sensitivity, specificity, simplicity, rapidity, automation and miniaturization. Other areas of study include developing simple, rapid sample preparation techniques together with evaluation of candidate gene probe methods for the analysis of test analytes in environmental samples such as air, water and soil.

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INTRODUCTION

Gene probe assays are an increasingly important technology in detection and identification of biological agents, as well as medical diagnostics. Gene probe assays use discrete sequences of nucleic acid (DNA or RNA) that bind to complementary nucleic acid sequences (RNA or DNA) in a sequence specific manner, through the process of hybridization. Since nucleic acid is a component of all living material, gene probes can be devised and used against nucleic acid from essentially all sources including viruses, bacteria, plants or animals. Gene probes can be used for identifying organisms at almost all levels of the taxonomic classification level (family, order, genus, species and strain). They can be generic when directed at highly conserved sequences that cross different classes of organisms or they can be highly specific. In a BW context, gene probes are usually directed against a virulence factor or some signature sequence which distinguishes it from related non-pathogenic strains. While gene probes can be used to detect the presence of and indicate the identity of conventional biological agents (eg. viruses and bacteria), they can also be used to identify genes that have been transferred naturally or by human design into foreign vehicles (eg. cloning of BW toxin genes into innocuous organisms or liposome-encapsulation of infectious viral nucleic acid). While gene probes cannot be used to detect toxins directly, it is possible to detect signature genes or fragments thereof, if present in a sample, for example, a crude toxin preparation.

We are continuing to evaluate and gain experience with gene probe assays in terms of probe design strategies, assay formats and assay sensitivities with the goal of developing identification strategies that are rapid, simple, sensitive and specific. Much of this work has been done using Newcastle disease virus (NDV) as a model. NDV was chosen as a model for two reasons. First, NDV has been developed as a BW viral simulant for use in field experiments at DRES [1]. Second, many BW viral threat agents are RNA viruses. Since the genomic nucleic acid component of NDV is RNA, NDV serves as a useful model for developing detection methods for RNA viruses.

We have previously developed a non-radioactive, colorimetric gene probe assay for NDV using two different solid supports, namely nylon membranes [2] and polystyrene mictrotiter plates [3]. The gene probe used in each of these assays was a 673 bp DNA fragment of the major nucleocapsid protein gene. The 673 bp sequence was labelled with digoxigenin, a non-radioactive label, and used as a probe against unlabelled 673 bp DNA. This assay format is termed a direct assay since the bound target DNA is hybridized directly with a gene probe. An alternative assay format, called a sandwich assay, has been described in the literature [4]. This approach uses two probe sequences. The first probe sequence is a single-stranded recombinant M13 vector DNA molecule containing a cloned "target specific" gene probe sequence. This recombinant probe sequence recognizes and binds to the target analyte. The second probe is linear, double stranded M13 sequence DNA that recognizes and binds to the "M13 sequence" of the first probe. The implication of the sandwich assay format is that it may allow for enhanced assay sensitivity through the formation of networks (multi-labelled complexes) brought about through the overlapping of complementary regions of the two gene probes. This study compares a sandwich assay format to a direct assay format for NDV. The sandwich assay described in this study was similar in design to the sandwich assay described above except that we used, as the primary probe, double-stranded M13 recombinant DNA that was linearized and denatured to generate complementary single-stranded sequences instead of circular single-stranded recombinant DNA.

MATERIALS AND METHODS

Reagents   Common reagents used in this study such as bovine serum albumin (BSA), bromophenol blue, ethidium bromide, ethylene diamine tetra-acetic acid (EDTA), glycerol, magnesium chloride, phenol, potassium chloride, sodium acetate, sodium chloride, sodium citrate, sodium dodecyl sulfate (SDS), sodium N-lauroylsarcosine, and Tris, were obtained from Sigma Chemical Co. (St. Louis, MO).   N-butanol was obtained from Caledon Laboratories Ltd. (Georgetown, ON).   Ethanol was purchased from Kodak International Biotechnologies Inc. (New Haven, CT).

NitroBlue Tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) used in the color development buffer, were obtained from Fisher-Promega (Edmonton, AB).

Low Melting Point (LMP) ultrapure agarose and ultrapure agarose were obtained from Gibco-BRL Life Technologies (Burlington, ON).

DNA molecular weight markers (λ phage DNA digested with *Hind* III restriction enzyme) and Sephadex G-50 were purchased from Pharmacia-LKB (Baie d'Urfe, Quebec).

SnaB I restriction enzyme and its commercially supplied buffer (buffer M), random primer digoxigenin labelling kit for labelling gene probes with digoxigenin, α-digoxigenin-alkaline phosphatase (α-dig-AP) and control digoxigenin-labelled pBR328 DNA were purchased from Boehringer Mannheim Company (BMC, Laval, Quebec).

PCR reaction components (other than PCR primers) were obtained from Perkin-Elmer Applied Biosystems Canada (Mississauga, ON).

PCR primers, NDVNP-PR5 (5' OH-acagagaattcgtaagttac-OH 3') and NDVNP-PR6 (5' OH-gctgtctcatctgcagtcat-OH 3'), were synthesized by the Regional DNA Synthesis Laboratory of the University of Calgary (Calgary, AB). Oligo^{TM} Primer Analysis Software program version 4.1 from National Biosciences (Plymouth, MN) was used for primer design. Restriction enzyme site selection and sequence analysis was performed using PC Gene version 6.5 software from Intelligenetics Incorporated (Mountainview, CA).

All water used in this study was either triple distilled, deionized water which was made sterile by autoclaving or nuclease-free water from Promega-Fisher Scientific (Edmonton, AB).

Buffers   1x *SnaB* I restriction enzyme buffer (Buffer M):

10 mM Tris-HCl (pH 7.5 at 37°C), 10 mM MgCl$_2$, 50 mM NaCl, 1 mM dithioerythritol

1x PCR buffer

50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM MgCl$_2$, 0.01% (w/v) gelatin, 200 µM of each of dATP;dTTP;dCTP;dGTP, 0.2 µM NDVNP-PR5 primer, 0.2 µM NDVNP-PR6 primer, 0.025 units/µL of Amplitaq^{TM} enzyme

10x gel loading buffer

0.1% bromophenol blue, 1.0% SDS, 0.1 M EDTA (pH 8.0), 50% glycerol

1x TAE buffer

40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, (pH 7.2)

1x TE buffer

10 mM Tris-HCl, 1 mM EDTA (pH 7.5)

10x hexanucleotide buffer

62.5 A$_{260}$ Units/mL random hexamers in 2 mg/mL BSA, 0.5 M Tris-HCl, 0.1 M MgCl$_2$, 1 mM dithioerythritol, (pH 7.2 at 20°C)

10x dig-dNTP buffer

1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM digoxigenin-dUTP, (pH 6.5 at 20°C)

1x SSC

> 0.15 M sodium chloride, 0.15 M sodium citrate buffer (pH 7.0)

Hybridization buffer

> 5x SSC, 0.1% Na-salt N-lauroylsarcosine, 0.02% SDS, 1% BMC
>
> blocking reagent^{TM}

Assay buffer 1

> 2x SSC, 0.1% SDS (pH 7.8)

Assay buffer 2

> 0.1x SSC, 0.1% SDS (pH 8.0)

Assay buffer 3

> 100 mM Tris, 150 mM NaCl (pH 7.5)

Assay buffer 4

> 100 mM Tris, 150 mM NaCl, 0.5% BMC blocking reagent^{TM} (pH 7.5)

Assay buffer 5

> 100 mM Tris, 150 mM NaCl, 0.5% BMC blocking reagent^{TM} (pH 7.5),
>
> anti-digoxigenin-alkaline phosphatase Fab conjugate at a concentration
>
> of 300 mU/mL (1/2500 dilution of commercial stock)

Assay buffer 6

> 100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$ (pH 9.5)

Color development solution

> 100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$ (pH 9.5), 0.34 mg/mL NBT
>
> and 0.17 mg/mL BCIP

Assay buffer 7

10 mM Tris, 1 mM EDTA (pH 8.0).

Methods *Preparation of Target DNA*

Target analyte used in this study was a 673 bp double-stranded DNA fragment of the major nucleocapsid protein gene of Newcastle disease virus. The target DNA was prepared by PCR amplification of a previously constructed recombinant M13 bacteriophage vector molecule containing the 673 bp gene fragment [5].

Recombinant vector DNA was isolated from *E.coli* DH5αF' cells and purified according to the large scale alkaline lysis plasmid purification procedure described elsewhere [5]. Recombinant vector DNA was then linearized with *SnaB* I restriction enzyme. *SnaB* I digestions were performed in 1x buffer M. Final concentrations were typically 10-50 ng/μL DNA and 0.05 to 0.5 units/μL of *SnaB* I enzyme. Digestions were carried out for 1-2 hours at 37°C. Then, 10 μL of 1 ng/μL concentration of linearized recombinant DNA was used as the template in a PCR reaction and added to a 90 μL aliquot of a 1.1x PCR buffer master mix to give a final 1x PCR buffer concentration in sterile 1.5 mL "oil-less" PCR tubes (Bio/Can, Mississauga, Ontario). Three negative control samples (20 μL total volumes) were run in parallel, in which water was used in place of template DNA. The tubes were placed into a pre-warmed thermocycler (Perkin-Elmer, Mississauga, Ontario) at 94°C for 5 min and the cycler was then run based on the following program: 94°C (1 min), 47°C (2 min), 72°C (3 min) per cycle for 30 cycles followed by 72°C (7 min).

Samples and controls were analyzed by horizontal agarose gel electrophoresis against molecular weight standards (λ-Hind III DNA) for the presence or absence of the 673 bp amplified product. A 2 μL aliquot from each tube was mixed with a 2 μL of 10x gel loading buffer and brought to a total volume of 20 μL with water. Ten μL of this mixture was loaded onto a 1% agarose gel (0.75 g of agarose in 75 mL of 1x TAE buffer) containing 0.5 μg/

mL ethidium bromide. The samples were subjected to horizontal agarose gel electrophoresis under the conditions of 50-60 V for 1-2 h at room temperature in 1x TAE buffer without ethidium bromide. The gel was visualized by UV illumination on a Fotodyne^{TM} UV light box (Bio/Can Scientific, Mississauga, ON). The sample tubes containing amplified product were combined and subjected to horizontal agarose gel electrophoresis under the conditions of 50-60 V for 2-3 h at 4°C through a 2% low melting point agarose gel in 1x TAE buffer. The gel was briefly exposed to UV light (prep setting) to visualize the amplified 673 bp band for excision. The band was excised from the gel and placed into 1.5 mL microcentrifuge tubes. A 1/3 volume of 1x TE buffer was added, based on an estimation of the volume of the excised gel material using a specific density of 1.0 g/mL. The tubes were placed in a 65°C waterbath for 15 min to melt the agarose. The tubes were cooled to room temperature for 2-5 min. A 1/3 volume of water-saturated phenol was added, the tube was vortexed for 30 sec and centrifuged at 16,000 x g for 5 min. The top aqueous layer was transferred to new tubes and subjected to a second extraction with water-saturated phenol. The aqueous layer was removed and then extracted several times with an equal volume of dry n-butanol (vortexed for 30 sec and centrifuged for 1 min at 16,000 x g for each extraction). The extractions were repeated until the total volume of the aqueous layer was 1/10 the starting volume. The DNA was precipitated from the aqueous layer by adding a 1/10 volume of 3M sodium acetate (pH 5.0) and 2 volumes of 95% ethanol, followed by incubation at -20°C for 1 h or at -70°C for 30 min. The precipitate was pelleted by centrifugation at 16,000 x g for 30 min. The pellet was washed once with 70% ethanol, vacuum-dried (10-30 min) and finally re-suspended in TE buffer or nuclease-free water. The DNA was analyzed for quality and quantity by running a sample on a regular 1% agarose gel at room temperature using methods described above. The concentration of sample DNA was obtained by comparing band intensities to molecular weight markers of known concentration (λ DNA digested with Hind III).

Preparation of Probe DNA for the Direct Assay

The gene probe used in the direct assay was PCR amplified 673 bp double-stranded DNA, labelled with digoxigenin using a commercial random primer digoxigenin labelling kit. Briefly, the DNA was heat denatured for 10 min in a dry temperature bath at 100°C and placed on ice for 5 min. Ninety μL of the denatured DNA was added to 24 μL of 10x hexanucleotide buffer, 24 μL of 10x dig-dNTP buffer, 0.1 Units/μL Klenow enzyme, and made to a total volume of 240 μL with water. The final DNA concentration in the labelling reaction mixture was between 20 and 50 ng/μL. The labelling reaction was carried out at 37°C for 16 h. The reaction was quenched by adding 0.5 M EDTA (pH 8.0) to a final concentration of 0.02 M. The samples were then passed through Sephadex G-50 spin columns to purify the probe-labelled material. The concentration of the digoxigenin-labelled probe material was estimated by dot blot analysis in which intensities of diluted samples were compared against dilutions of control dig-pBR328 DNA based on procedures described in the probe labelling kit from the manufacturer. A detailed description for probe quantitation is the same as that described below for the direct assay, except that the labelled DNA was spotted onto the membrane in place of target DNA and the pre-hybridization and hybridization steps were not required.

Preparation of Probe DNA for the Sandwich Assay

The primary probe used in the sandwich assay was *SnaB* I linearized recombinant vector DNA (described earlier). The secondary probe was *SnaB* I linearized M13mp18 vector DNA that was labelled with digoxigenin and quantitated in the same manner as described above.

Detection of Target NDV Sequences by DNA Hybridization

The procedures for hybridization and detection of target DNA with digoxigenin-labelled probes were based on the protocols provided in the digoxigenin random primer labelling and detection kit from Boehringer Mannheim Canada (Laval, Quebec) and vary slightly depending on which assay was used. The procedures are outlined below.

Direct Assay

Concentrated stocks of double-stranded target DNA were denatured to single strands by heating at 100°C for 10 min followed by chilling on ice for 5 min. Dilutions of the denatured target DNA were prepared with water. Prior to spotting sample onto Hybond[TM] nylon membranes (Amersham Canada, Oakville, ON), the membranes were pre-wetted with 200 µL of water per well under vacuum using a 96 well vacuum filtration manifold. Then, 10 µL of each DNA sample were applied per well. Samples were spotted in triplicate. After spotting the samples, the membranes were removed from the vacuum filtration manifold and allowed to air dry for 20-30 min. The membranes were exposed to UV light (preparative setting) for 5 min by placing membranes face down on a transillumination UV light table (Fotodyne[TM]) to cross-link DNA to the membrane. The membranes were then placed into hybridization bags containing 0.2 mL/cm^2 hybridization buffer (without probe) and incubated in a water bath shaker at the hybridization temperature of interest for 1-2 h to block un-reacted DNA binding sites on the membrane. The buffer was removed and replaced with 0.05 mL/cm^2 of fresh hybridization buffer containing digoxigenin-labelled 673 bp DNA (probe material was always denatured by heating at 100°C for 10 min and chilling on ice for 5 min beforehand). The membranes were incubated at 68°C overnight (16-20 hr) in a shaking water bath. The membranes were then washed 2x for 5 min in 0.5 mL/cm^2 of assay buffer 1 at room temperature to remove unbound probe. This was followed by two 5-15 min washes in 0.5 mL/cm^2 of assay buffer 2 at temperatures at or above room temperature. All subsequent washes were done at room temperature. The membranes were prepared for detection by first rinsing briefly (1 min) in assay buffer 3. This was followed by washing in 1 mL/cm^2 of assay buffer 4 for 30 min and then 1 mL/cm^2 of assay buffer 5 for 30 min. This was followed by two washes in 1 mL/cm^2 of assay buffer 3 for 15 min each and then equilibration in 1 mL/cm^2 of assay buffer 6 for 5 min. The membranes

were incubated in the dark with 0.1 mL/cm^2 of color development solution. Color development was monitored and allowed to proceed anywhere from 1 to 24 hours at which time the reaction was quenched by removing the membrane from the color development solution and washing the membrane in 1 mL/cm^2 of assay buffer 7.

Sandwich Assay (One step)

In the one step sandwich assay, the primary probe (*SnaB* I linearized recombinant M13mp18 DNA) and the secondary probe (linear, digoxigenin-labelled M13mp18 DNA) were added together in a single hybridization step. Other than this, the hybridization and detection procedures were the same as those described for the direct assay.

Sandwich Assay (Two step)

In the two step sandwich assay, the primary and secondary probes were added in separate hybridization steps. Heat-denatured primary probe was added first and allowed to hybridize. The membranes were then washed twice for 5 min at room temperature with 0.5 mL/cm^2 of assay buffer 1 followed by two washes for 15 min at room temperature with 0.5 mL/cm^2 of assay buffer 2. The secondary probe was added and allowed to hybridize under the same conditions as the primary probe. The remaining steps were the same as described for the direct assay.

RESULTS

A schematic diagram of a direct assay and a sandwich assay compared in this study are presented in Figure 1. The distinction between the two assays was the way in which the 673 bp NDV sequence was used as a probe for target DNA. In the direct assay format, the target NDV DNA was detected directly using a digoxigenin-labelled 673 bp probe. For the sandwich assay, the target

DNA was detected by hybridization with a primary probe (the 673 bp probe sequence contained within M13mp18 recombinant molecule), which in turn was detected by hybridization with a secondary probe (digoxigenin-labelled M13mp18 DNA). Prior to comparing these two assay formats, we decided to determine if the hybridization reaction for the sandwich assay could be performed in one step (both probes at the same time) or if separate hybridization reactions were required. The advantage to the former option is that it would reduce the assay time significantly as both probes would be allowed to hybridize during the same step. A comparison of the two assays (data not shown), showed no significant differences in sensitivity. Consequently, the single-step sandwich assay was chosen for further comparisons against the direct assay format.

In order to make a comparable assessment of the relative sensitivities of the direct assay and the sandwich assay, it was necessary to use equivalent molar probe concentrations. Figure 2 shows the results of a comparison between the direct assay and the sandwich assay when a molar probe concentration of approximately 20 pM was used for both assays. The lower detection limits for the direct and sandwich assays were found to be the same (0.4 pg or 6×10^5 molecules of target DNA).

To ensure that the probe concentrations in the sandwich assay were not limiting, they were each increased to approximately 30 pM. The results from this analysis are presented in Figure 3. We observed very high backgrounds and poorer detection limits for the sandwich assay. The high backgrounds on the membrane made it difficult to estimate the lower detection limit accurately. In an effort to reduce the background problem, we reduced the concentration of the secondary probe in the sandwich assay to 3 pM while maintaining the primary probe concentration at 30 pM (Figure 4). The background problem improved some, but detection limits were well below those of the direct assay. Further attempts to improve sensitivity of the sandwich assay by altering the probe concentrations failed to improve detection limits any further.

DISCUSSION

In this paper, the sandwich assay format was compared to the direct assay format. Prior to comparing these two formats, we were able to show that for the sandwich assay, there was no difference in terms of sensitivity when applying the probes separately in two hybridization steps or together in one hybridization step. As a consequence, the single-step sandwich assay was used in comparison studies with the direct assay since it required less time to perform.

In order to compare the two assays in an equivalent manner, roughly equivalent molar concentrations of the "673 bp sequence" were used. The sandwich assay was found to be no more sensitive than the direct assay under these conditions. One possible explanation for this may be that overlapping complementary regions were not formed under the conditions used. For example, the network effect is described in the literature for probes prepared by "nick-translation" [4], a process whereby probe molecules of various random lengths are generated during the labelling reaction as a consequence of random enzymatic cleavage. The probe labelling method used in our study was the "random-primer" method in which random primer sequences are used to generate labelled probe sequences. Perhaps the random-primer method does not generate as many overlapping complementary probe molecules with which to form stable networks. In addition, it has been suggested that the formation of networks is accelerated by including dextran sulfate 500 in the reaction [6]. Our experiments did not include the use of dextran sulfate. Another reason that we did not achieve lower detection limits for the sandwich assay, may be that the recombinant probe, being roughly 12 times larger than the 673 bp probe, masked neighbouring DNA target molecules or was sterically hindered from binding to all available target DNA molecules as a consequence of its larger size. This in turn, may have resulted in fewer primary probe/target hybridized complexes and thus poorer than expected detection limits.
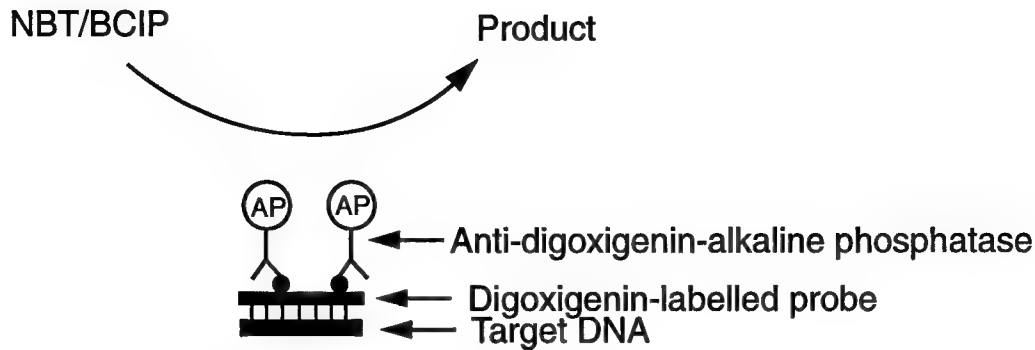
When we tried to improve sensitivity limits of the sandwich assay by increasing the probe concentration, we experienced significant background problems. This background was due to non-specific, irreversible binding of the probe(s) to the filter as a consequence of high concentrations and also most likely as a consequence of the length of the primary probe (it has been suggested that longer probes have been related to higher backgrounds [4]).

Other drawbacks to the sandwich assay include the fact that 10x more probe material was required for each of the probes to achieve detection limits similar to those demonstrated for the direct assay. This, in turn, increases the costs associated with probe labelling reactions. Finally, a major disadvantage to the sandwich assay was that it was more technically complicated, in that much more effort was required for probe preparation and probe design. Based on these findings, the direct assay is the better assay.

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Direct Assay

NBT/BCIP → Product

(AP) (AP) ← Anti-digoxigenin-alkaline phosphatase
← Digoxigenin-labelled probe
← Target DNA

Sandwich Assay

NBT/BCIP → Product

(AP) ← Anti-digoxigenin-alkaline phosphatase
← Digoxigenin-labelled secondary probe(M13 DNA)
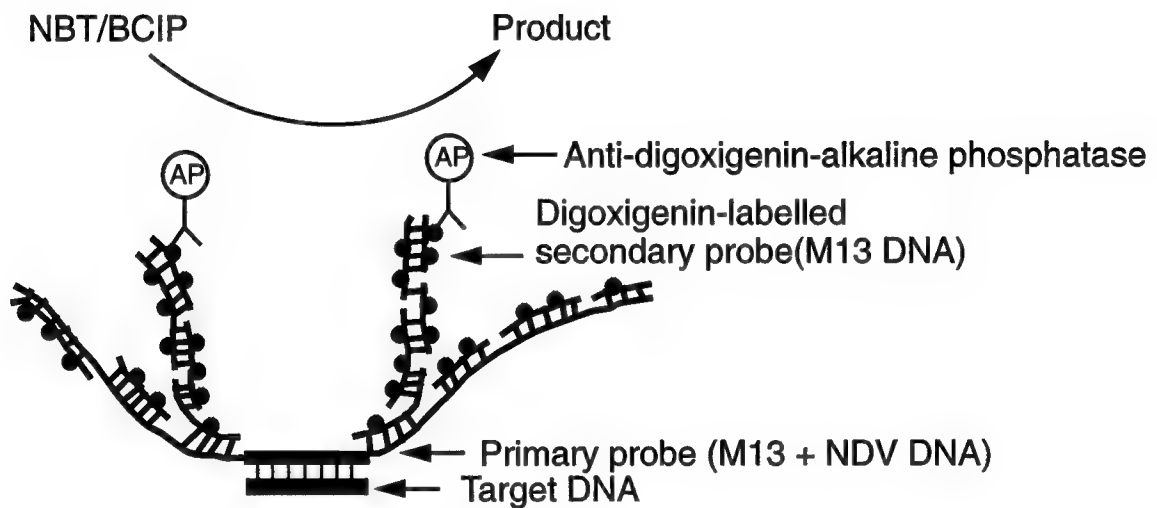← Primary probe (M13 + NDV DNA)
← Target DNA

Figure 1

Schematic diagram of a direct assay and a sandwich assay. In each assay the 673 bp fragment of the NDV NP gene is used as a "target-specific" probe. In the direct assay, the 673 bp sequence is labelled with digoxigenin and binds to the target DNA (673 bp fragment of the NDV NP gene). In the sandwich assay, the primary probe is the 673 bp sequence cloned into M13 viral DNA which binds to the target DNA. The secondary probe, digoxigenin-labelled M13 DNA, binds to the M13 sequences of the primary probe forming an overlapping network effect through hybridization of complementary sequences.
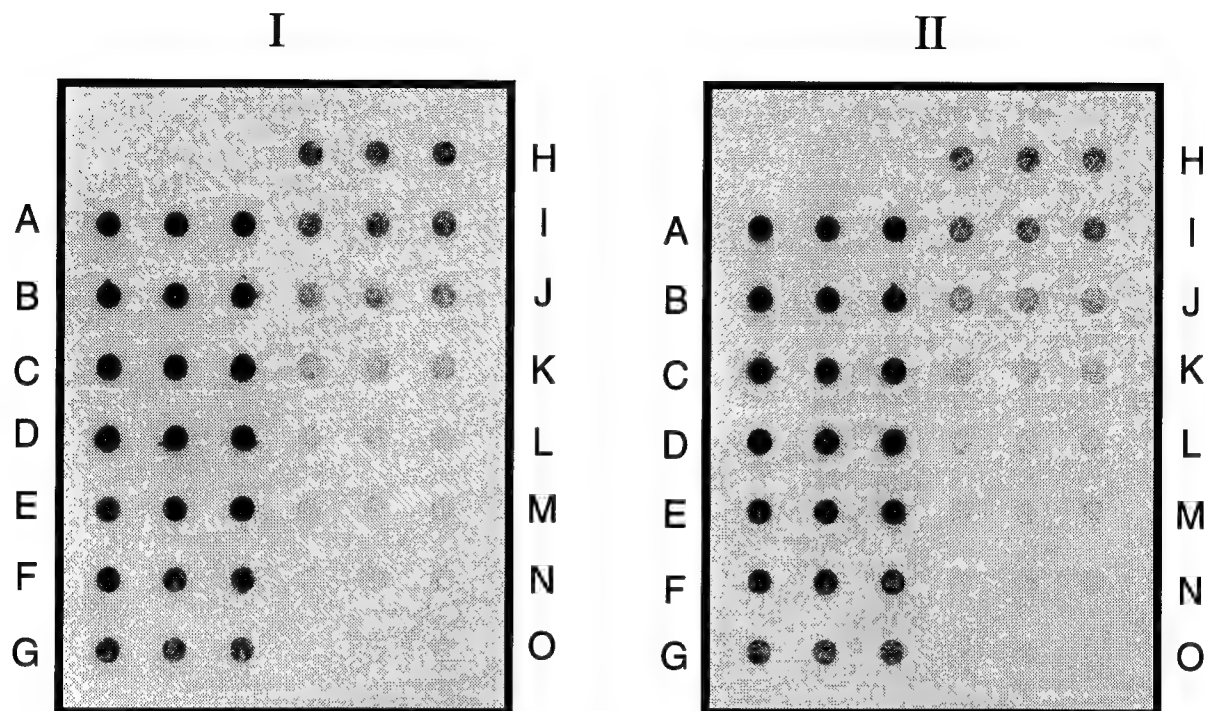
Figure 2

Comparison of a direct and a sandwich assay-experiment 1. **I.** Direct assay using a probe concentration of 23 pM (10 ng/mL). **II.** Sandwich assay using primary probe concentration of 20 pM (100 ng/mL) and a secondary probe concentration of 21 pM (100 ng/mL). Target DNA is spotted in triplicate for each membrane as follows. B: 100 pg C: 80 pg D: 60 pg E: 40 pg F: 20 pg G: 10 pg H: 8 pg I: 6 pg J:4 pg K: 2 pg L: 1 pg M: 0.8 pg N: 0.6 pg O: 0.4 pg. Digoxigenin-NDV673 probe (+ve control) is spotted in triplicate for each membrane in row A (100 pg).

Conditions include 68°C hybridization for 20 h, 2x15 min washes at room temperature in 0.1x SSC/0.1% SDS, 20 h color development.
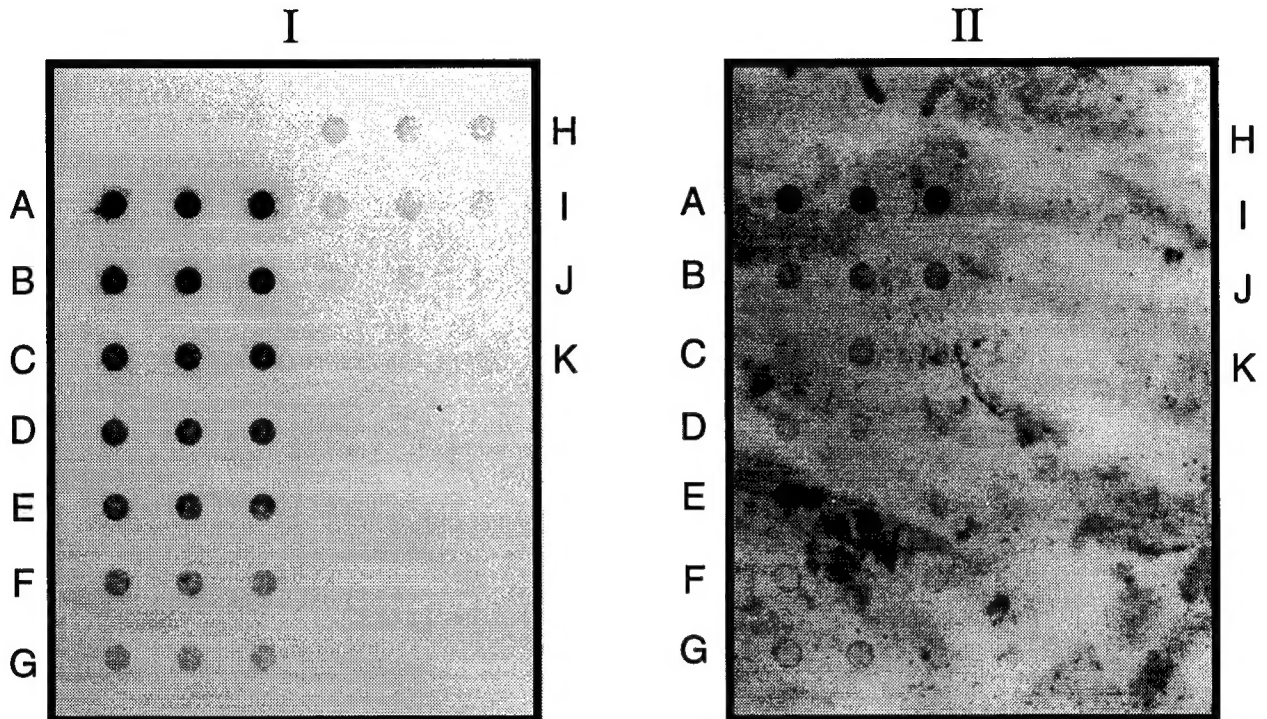
Figure 3

Comparison of a direct and a sandwich assay-experiment 2. **I.** Direct assay using a probe concentration of 23 pM (10 ng/mL). **II.** Sandwich assay using primary probe concentration of 29 pM (148 ng/mL) and a secondary probe concentration of 31 pM (144 ng/mL). Target DNA is spotted in triplicate for both membranes as follows. B: 100 pg C: 50 pg D: 25 pg E: 12.5 pg F: 6.25 pg G: 3.12 pg H: 1.6 pg I: 0.8 pg J: 0.4 pg K: 0.2 pg. Digoxigenin-NDV673 probe (+ve control) is spotted in triplicate for both membranes in row A (100 pg). Conditions include 68°C hybridization for 20 h, 2x15 min washes at room temperature in 0.1x SSC/0.1% SDS, 20 h color development.
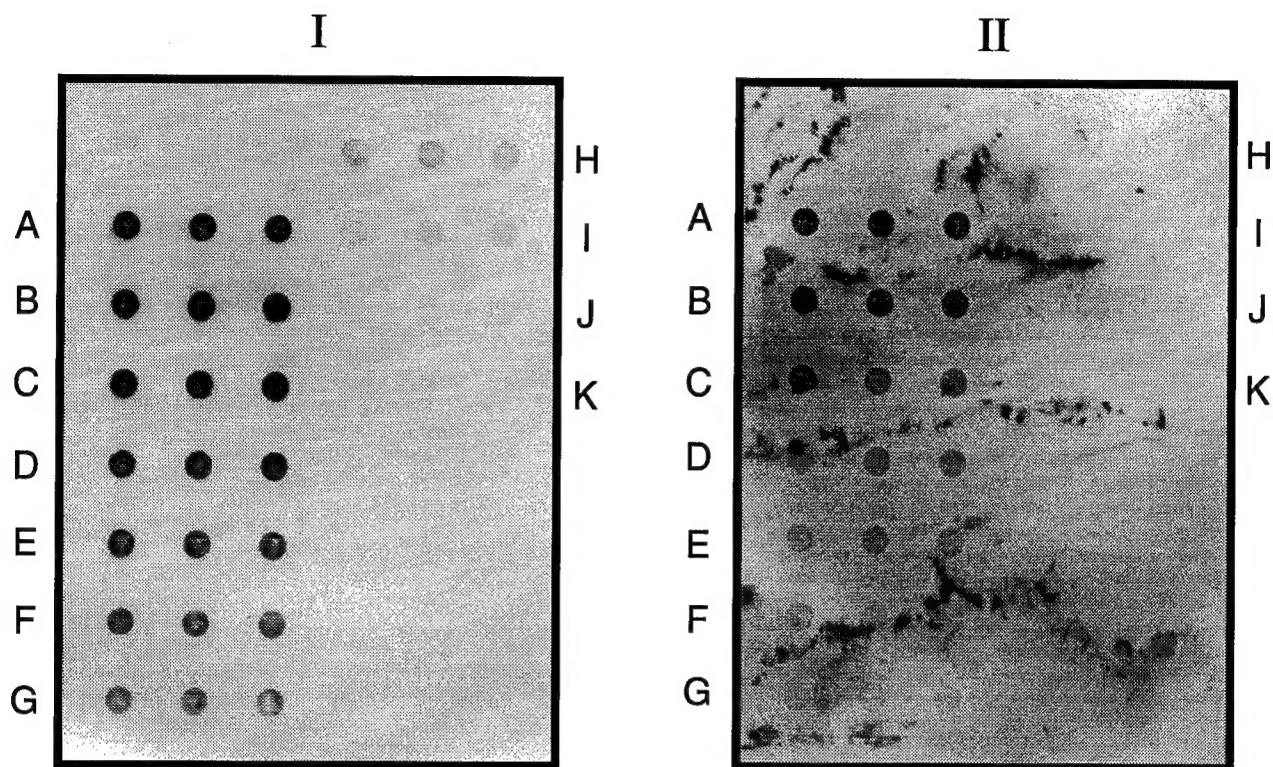
Figure 4

Comparison of a direct and a sandwich assay-experiment 3. **I.** Direct assay using a probe concentration of 23 pM (10 ng/mL). **II.** Sandwich assay using primary probe concentration of 29 pM (148 ng/mL) and a secondary probe concentration of 3.1 pM (14.4 ng/mL). Target DNA is spotted in triplicate for both membranes as follows. B: 100 pg C: 50 pg D: 25 pg E: 12.5 pg F: 6.25 pg G: 3.12 pg H: 1.6 pg I: 0.8 pg J: 0.4 pg K: 0.2 pg. Digoxigenin-NDV673 probe (+ve control) is spotted in triplicate for both membranes in row A (100 pg). Conditions include 68°C hybridization for 20 h, 2x15 min washes at room temperature in 0.1x SSC/0.1% SDS, 20 h color development.

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A sandwich gene probe assay was evaluated in comparison to a direct gene probe assay. The target sequence used in each of the assays was a 673 bp DNA fragment of the major nucleocapsid protein gene of NDV. In the direct probe assay, the 673 bp DNA fragment was labelled with digoxigenin and hybridized to unlabelled 673 bp target DNA. In the sandwich assay, the target DNA was detected using two probes. The primary probe was unlabelled, recombinant M13mp18 viral DNA containing the 673 bp gene fragment which hybridized to the 673 bp target DNA. The secondary probe was digoxigenin-labelled M13mp18 DNA which hybridized to the M13 sequences within the primary probe.

The sandwich assay resulted in detection limits similar to those demonstrated for the direct assay (10^5 molecules of purified target DNA) when molar probe concentrations for the two assays were around 20 pM. When molar probe concentrations in the sandwich assay were increased beyond this, sensitivity decreased and background problems due to non-specific binding became evident. Based on these results, the direct assay is the method of choice since the sandwich assay was no more sensitive than the direct assay, required more probe material and required additional time-consuming probe preparation steps.

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Gene Probes

Nucleic Acid Hybridization

Direct Assay

Sandwich Assay